PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	Confirmation No. 7618
Makiko FLISS *et al.*	Group Art Unit: 1637
Application Serial No.: 10/601,692	Examiner: Jeffrey Fredman
Filed: June 24, 2003	
For: MITOCHONDRIAL DOSIMETER	Atty. Dkt No.: 001107.00357

DECLARATION UNDER RULE 132

I, Anirban Maitra, declare:

1. I am an Associate Professor of Gastrointestinal Pathology and Oncology at the Johns Hopkins University School of Medicine, Baltimore, MD. I am currently an affiliate of the McKusick-Nathans Institute of Genetic Medicine as well as an Editor-in-Chief of the journal *Current Molecular Medicine.*

2. I have reviewed U.S. Patent Application Serial No. 10/601,692.

3. Mutations are typically defined with reference to a wild-type allele. "A wild-type allele is the normal, as opposed to the mutant, gene or allele." See Genetics Home Reference at the National Library of Medicine, National Institutes of Health website; Exhibit 1. The specification teaches, "Mitochondrial mutations are determined with reference to wild-type human mitochondrial sequence. Sequence information can be found at the website gen.emory.edu/mitomap.html and at SEQ ID NO: 1." Page 8, lines

18-20. Therefore, a mutation described in the specification would be defined with reference to the wild-type reference sequence disclosed as SEQ ID NO: 1.

4. The application mentions a mutation designated "ΔC at nucleotide 302" of the human mitochondrial DNA. See specification, for example, at page 3, line 12. I, as a skilled practitioner in the art of human genetics, would have recognized that this description of a mutation is an error because a ΔC mutation at nucleotide 302 could not possibly occur. A ΔC mutation means a deletion of a cytidine monophosphate nucleotide from the wild-type sequence. However, the wild-type reference sequence shown in the application indicates that an adenosine monophosphate resides at nucleotide position 302. Therefore, a ΔC mutation at this position must be an error. I believe that any person of ordinary skill in human genetics would have recognized this designation as an error.

5. Upon noting the erroneous ΔC mutation at nucleotide 302, I would have immediately noticed the run of C nucleotides directly adjacent to position 302 in SEQ ID NO: 1. In general, such runs are notorious for being deletion prone due to polymerase "slippage" during replication. Appelmelk and Vandenbroucke-Grauls teach, "DNA slippage in C-tracts may give rise to daughter DNA that is either one C shorter or longer." *Helicobacter pylori* Physiology and Genetics. Online textbook. Chapter VI, 35, at page 3, lines 29-31; Exhibit 2. This particular run of C nucleotides had already been noted in the scientific literature as polymorphic. See Marchington *et al*: ". . . the length of the D310 tract can vary between individuals." *Am. J. Hum. Genet.* 1997:60, 408-416, at page 410, right hand column, lines 45-46; Exhibit 3. I would have realized, based on the general properties of C nucleotide tracts and the particular polymorphic nature of this C nucleotide tract taught in the scientific literature, that the appropriate correction of the

error within the originally filed specification would have been that the ΔC mutation was at nucleotide 303.

7. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: June 05, 2007

Anirban Maitra

Enclosures:

Exhibit 1: Definition of "wild-type allele." Genetics Home Reference at the National Library of Medicine, National Institutes of Health website. ghr.nlm.nih.gov/

Exhibit 2: Marchington *et al.*, *Am. J. Hum. Genet.* 1997:60, 408-416.

Exhibit 3: Appelmelk and Vandenbroucke-Grauls, *Helicobacter pylori* Physiology and Genetics. Online textbook. Chapter VI, 35.

EXHIBIT 1

Wild-type allele

Definition(s)

- The normal, as opposed to the mutant, gene or allele

  Definition from: GeneTests ⇨ from the University of Washington and Children's Health System, Seattle

Published: May 29, 2007

EXHIBIT 2

Helicobacter pylori
Physiology and Genetics
Edited by Harry L.T. Mobley, George L. Mendz, and Stuart L. Hazell
ASM PRESS

35. Lipopolysaccharide Lewis Antigens

Ben J. Appelmelk
Christina M. J. E. Vandenbroucke-Grauls

Department of Medical Microbiology Vrije Universiteit Medical School, van der Boechorststraat 7 1081, BT Amsterdam, The Netherlands

Helicobacter pylori
Neisseria gonorrhoeae

As determined by serological techniques, the O-antigen of lipopolysaccharide (LPS) of more than 80% of *Helicobacter pylori* strains tested worldwide express Lewis blood group antigens (36, 60, 71). This percentage possibly represents an underestimation; it was demonstrated that some *H. pylori* strains do not react with anti-Lewis x (Le^x) monoclonal antibodies (MAbs) while structurally they were shown to express Le^x (39). Thus, Lewis antigen expression in *H. pylori* is highly conserved. This restricted diversity in O-antigen structure is striking, and the question arises whether *H. pylori* Lewis antigens play a role in pathogenesis. An analogous situation is found in *Neisseria gonorrhoeae*, where conserved LPS O-antigen epitopes directly interact with the host via ligand-lectin binding (35).

H. pylori
Haemophilus influenzae
Campylobacter jejuni

There are additional reasons why *H. pylori* LPS Lewis antigens are thought to play a role in pathogenesis beyond merely providing length to the LPS (although length itself already contributes to virulence) (7). (i) *H. pylori* LPS displays phase variation, defined as the high frequency of reversible change of LPS phenotype (2, 5, 68, 69). In other bacteria (*Neisseria* spp. and *Haemophilus influenzae*), phase variation of LPS is crucial to virulence (37, 65). (ii) *H. pylori* LPS displays molecular mimicry with the host (4). Gastric human epithelial cells also express $Le^{x/y}$ blood group antigens. The expression by microorganisms of surface structures similar to those found in the host is called molecular mimicry. Examples of other pathogens displaying molecular mimicry are *Campylobacter jejuni* and *Neisseria* spp. (33). The role of mimicry in pathogenesis can be twofold. (a) *H. pylori* mimicry is pathogenic. Infection might break tolerance to the shared epitopes and induce autoantibodies. Bound antibodies may induce tissue damage, for instance, by fixing complement. (b) Molecular mimicry might provide immune escape by preventing the formation of antibodies directed to the epitopes shared by self and microorganism; the lack of response to a surface-located antigen might

contribute to persistence of infection. (iii) *H. pylori* Lewis antigens might interact with host lectins. Several host lectins are known to interact with host Lewis antigens (22, 42); the same lectins may interact with *H. pylori* Lewis antigens. Such interaction may have biological consequences such as bacterial adhesion, colonization, and cytokine induction.

In this chapter, we will discuss phase variation of *H. pylori* LPS, including LPS biosynthesis and genetics; the biological significance of Lewis antigen mimicry; and the role of Lewis antigens in interactions of *H. pylori* with host lectins. H. pylori

Phase Variation in *H. pylori* LPS

The structures of LPS isolated from a variety of *H. pylori* strains have been determined chemically. The overall architecture of *H. pylori* LPS is similar to that of LPS of other gram-negative pathogens. The lipid A moiety is connected to the oligosaccharide core region that in turn is connected to the O-antigen (or Lewis antigen). In many strains, the O-antigen consists of Le^x and/or Le^y (Table 1), but other blood group antigens (H type 1, Le^a, Le^b, nonfucosylated polylactosamine [=i-antigen], sialyl Lewis x, blood group A) have also been found (10, 11, 46, 47, 49). Strains expressing H type 2 have not been identified. Often, strains express more than one Lewis antigen (Table 2). For example, strain NCTC 11637 (ATCC 43504) expresses polymeric Le^x with n up to 8 or 9 that is substituted terminally in nonstoichiometric amounts with Le^y or H type 1. H. pylori

Phase Variation

Phase variation is defined as the random switching of LPS phenotype at frequencies that are much higher (sometimes >1%) than classical mutation rates. This process results in reversible loss and gain of certain LPS epitopes and results in a bacterial population that is heterogeneous with regard to LPS expression. Phase variation contributes to virulence by generating heterogeneity; certain environmental or host pressures select those bacteria that express the best adapted phenotype. An example is LPS sialylation in *Neisseria* spp. While nonsialylated bacteria are adherent and invasive, they are sensitive to the lytic action of serum; in contrast, sialylated bacteria adhere less well but are more resistant to serum (65). Phase variation allows outgrowth of nonsialylated bacteria during adhesion or invasion and of bacteria expressing sialylated LPS upon contact with serum.

Phase variation can be detected by colony-blotting with MAbs specific for LPS (5). An example is given in Fig. 1 where an *H. pylori* strain was probed with a MAb specific for H type 1. Three types of colonies are present: first, those that are completely reactive (dark colonies); the bacteria forming this colony originate from a single bacterial cell expressing H type 1, with no switching off to the H type 1-negative phenotype occurring during multiplication. Likewise, nonreactive colonies originate from a bacterial cell with a switched-off phenotype. Colonies with a dark sector originate from a cell with a switched-off phenotype that switched on during multiplication (often on more than one independent event per colony); clonal outgrowth of a switched-on variant gives rise to the sectors observed. By colony-blotting, many LPS phase H. pylori

variants were isolated from a single strain (NCTC 11637) (see Table 2).

Subsequently, variants were serotyped in enzyme-linked immunosorbent assay and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (Fig. 2, Table 2 and 3). The frequency of phase variation is in the range of 0.5 to 1%, but the frequency of switching on is not necessarily the same as switching off: the switch frequency of NCTC 11637 to variant 1b is in the 0.5 to 1% range, but the switch-back frequency to parent phenotype is only 0.07% (5). Phase variation is not restricted to laboratory strains; it also occurs in other strains including clinical isolates.

Molecular Mechanisms of LPS Phase Variation

The sequencing of the genome of two *H. pylori* strains has identified many LPS-related genes, including several glycosyltransferases potentially involved in phase variation (1, 12, 59, 62). Two similar but not identical α3-fucosyltransferse (α3-*fucT*) genes have been identified both in strain 26695 (HP0379 and HP0651) and in strain J99 (JHP1002 and JHP0596). Functional studies with the cloned and expressed gene products show that both FucT enzymes encoded by these two genes are able to form Le^x from lactosamine acceptors (31, 44). However, insertional mutagenesis studies have shown that they differ in fine-specificity (2). The HP0379-encoded α3-FucT has a preference for internal GlcNAc residues (i.e., not located at the nonreducing terminus) and yields polymeric Le^x, while HP0651-encoded α3FucT has a preference for terminal GlcNAc residues and forms mono/oligomeric Le^x. The HP0379-encoded α3-FucT can also function as an α4-FucT and can therefore also form $Le^{a/b}$ (3, 57).

HP0379
HP0651
JHP1002
JHP0596
H. pylori

HP0093/94 (JHP0086) is an α2-*fucT*; the gene product is required for biosynthesis of both Le^y and H type 1 (see below) (3, 67, 69). H type 2 epitopes do not occur in *H. pylori* LPS, and knocking out both α3-*fucT* genes in a strain that expresses $Le^{x/y}$ yields LPS that expresses i-antigen but no H type 2 (2). Thus, α3-fucosylation precedes α2-fucosylation. This was confirmed in enzyme assays with cloned α2FucT that forms Le^y from synthetic Le^x but not H type 2 from Galβ1→4GlcNAc (67). In contrast, this enzyme is able to form H type 1 with a Galβ1→3GlcNac acceptor.

HP0093
JHP0086
H. pylori

Sequencing of α2- and both α3-*fucT* genes revealed that they all carry long poly-C stretches close to the 5′ end of the gene. C-tracts are also present in LPS genes of *Neisseria* spp. and are a well-characterized cause of LPS phase variation (37). On replication, DNA slippage (slipped-strand mispairing) in C-tracts may give rise to daughter DNA that is either one C shorter or longer; this can occur at very high (1%) frequencies. The result is a high-frequency, reversible frameshifting. The consequence is a rapid on-off switching of enzyme activity. When a C-tract is present in the parent strain that to leads to a full-length, active gene product in the C + 1 or C − 1 daughters, the frameshifting will lead either to the production of nonsense polypeptides that

HP0651
HP0379
H. pylori

have no or little enzyme activity or, due to the occurrence of early stop codons, to a truncated inactive gene product. The molecular basis of phase variation in *H. pylori* was determined by sequencing the C-tracts in the α3-*fucT* genes of the parent strain (NCTC 11637) and in the phase variants (Table 3) (2). In the NCTC 11637 HP0651 is "off" due to the presence of a C9 tract; HP0379 is "on" in this strain (C10).

Phase variation from Le^x to i-Ag and back to Le^x

HP0651
HP0379

In the phase variant expressing i-Ag plus H type 1 (variant K4.1), both HP0651 (C9) and HP0379 (C11) are off; this explains the lack of Le^x and the biosynthesis of nonfucosylated polylactosamine (= i-antigen) in strain K4.1. In addition, K4.1 expresses H type I due to the presence of an active α2FucT. In strain K5.1, the Le^x-positive switch-back variant isolated from K4.1, HP0379 is "on" again (C10). Thus, phase variation from Le^x to i-Ag and back to Le^x can be understood at the molecular level through reversible length changes in the C-tract of α3-*fucT* gene HP0379, that is, from C10 to C11 and back to C10. A HP0379/HP0651-double knockout of strain 4187E (4187E-KO379/651) expresses a serotype identical to that of strain K4.1 (i.e., i-antigen and H type 1). Clinical isolate J233 expresses H type 1 plus i-Ag both as determined by structural chemistry (47) and by serology, and in that strain also both α3-*fucT* genes are off. We conclude that LPS serotype is determined by the on-off status of α3-*fucT*.

Phase variation from Le^x to Le^x plus Le^y

HP0379
HP0651

While strain NCTC 11637 expresses polymeric Le^x, H type 1, and a little Le^y, phase variant 1c strongly expresses both Le^x and Le^y. C-tract analysis shows that both HP0379 and HP0651 are "on" in strain 1c. Knockout studies in strain 4187E also show that the presence of an intact HP0651 is associated with a stronger Le^y expression and with reactivity with Mab 6H3 that recognizes monomeric Le^x. We conclude that HP0651 FucT preferentially fucosylates GlcNAc at the nonreducing terminus, thus forming an efficient acceptor for α2-FucT to form Le^y. In contrast, HP0379 α3-FucT would prefer internal GlcNAc, thus forming polymeric Le^x from the inside out, a structure that is evidently a less efficient acceptor. Consequently, as compared to variant 1c, less Le^y is formed in the parent strain.

Phase variation from Le^x to Le^y

HP0379
H. pylori

Variant 1b has a truncated LPS (Fig. 2) that strongly expresses Le^y (Table 2); this serotype is similar to that of strains MO19 and O6. Enzymatic analysis showed that this variant lacks GlcNAcT activity (5). The serotype of this strain can be explained by the following model. Likely there are two GlcNAcT enzymes, one that recognizes the core and adds the first GlcNAc and a second one that recognizes Gal and thus is responsible for chain elongation. Likely, the lack of GlcNAcT activity in variant 1b signifies lack of the second, elongating enzyme. Thus, first the core plus a single GlcNAc is formed in this variant. HP0379 is "on" in variant 1b, so that terminal Le^x is formed;

α2-FucT then forms Le^y. Although GlcNAcT genes have been identified in other species (13), they do not show significant homology with *H. pylori* open reading frames.

Phase variation forming Le^a

H. pylori
Escherichia coli

Variant 3a expresses polymeric Le^x plus Le^a (3). Hence, compared to NCTC 11637, this variant has lost both Le^y and H type 1. This can be explained by phase variation in α2-*fucT*, and indeed insertional inactivation of this gene in NCTC 11637 yields a mutant with a serotype indistinguishable from that of strain 3a (3). The α2-*fucT* gene also contains a C-tract and hence phase variation occurs along the lines sketched above for a α3-*fucT*. However, a second mechanism for phase variation was observed in the α2-*fucT* gene, namely a sequence (AAAAAAG) that allows mRNA slippage at the translational level (69). The result of this slippage is a −1 frameshift. The mechanisms involved are as follows: there are two anticodons for lysine, UUU and CUU. However, from the whole genome sequence it is known that *H. pylori* codes only for a $tRNA^{Lys}$ with the UUU anticodon while $tRNA^{Lys}$ with the CUU anticodon is missing. Hence, when AAG is encountered in the mRNA of α2*fucT*, the loaded $tRNA^{Lys}$ (UUU) slips one base back to allow the stronger interaction with AAA. This second mechanism may therefore compensate for 31 frameshifting due to C-tracts. These two mechanisms operate in the genome strain 26695. While this strain expresses Le^y (46), its α2-*fucT* gene is frameshifted (+1) due to the C-tract (62) and theoretically would yield an inactive α2FucT. However, presence of the translational −1 frameshift cassette AAAAAAG causes a −1 shift in the reading frame, an active enzyme to be formed and Le^y synthesis to take place. The mechanism of −1 slippage has been well investigated for the *dnaX* gene of *Escherichia coli* (29, 63).

Other phase variants

HP0178

Variant H11 expresses Le^x, Le^y, but no H type 1; hence, phase variation has to take place in the gene coding for β3-GalT (3). Variant D1.1 expresses a truncated LPS and does not react with any anti-Lewis MAb. This variant arose through phase variation from K4.1 through subsequent loss of the elongating GlcNacT (5). An sLe^x-expressing variant of P466 was isolated and characterized (46); *neuB* (HP0178), a gene required for biosynthesis of sialyl-Le^x, contains a C6-tract in strains 26695 and J99.

Biological Role of LPS Phase Variation

H. pylori

Is phase variation relevant in vivo? We isolated 30 *H. pylori* colonies from a single patient and found that 20% of the colonies expressed $Le^{x/y}$, while 80% expressed the i-Ag (8). By molecular typing, combined with C-tract sequencing, it was demonstrated that they are phase variants of the same strain. Thus, LPS phase variation contributes to strain diversity in vivo. The data shown above demonstrate that many of the currently known *H. pylori* LPS serotypes can be isolated as phase variants from a single strain (Table 2), and hence, theoretically any strain can express almost any LPS phenotype. Which factors determine the actual serotype expressed by a strain isolated from a clinical sample, or the distribution of serotypes

of multiple isolates obtained from a single patient? At present no single environmental or host factor has been identified that causes a change in LPS phenotype through selection of LPS phase variants. Prolonged growth of bacteria on solid agar leads to reversible loss of O-antigen (51), but whether phase variation is involved is not known.

The Biological Role of *H. pylori* Lewis Antigen Mimicry

H. pylori Mimicry Is Pathogenic

Mimicry can contribute to pathogenesis during infection due to *C. jejuni* (50). LPS of this bacterium expresses ganglioside structures similar to those occurring in nerve tissue. Upon infection, antiganglioside antibodies are formed that cause an autoimmune attack of peripheral nerves followed in some cases by paralysis (Guillain-Barré syndrome). Likewise, *H. pylori* LPS might induce anti-$Le^{x/y}$ antibodies that bind to the bacteria but also to the gastric epithelial cells; when followed by complement fixation this may lead to tissue injury (4). Indeed, immunization of mice with *H. pylori* induces anti-$Le^{x/y}$ MAbs that cross-react with gastric epithelium, in particular with gastric H^+,K^+-ATPase, the proton pump that is localized in the parietal cell canaliculi (Fig. 3) (4, 6). *H. pylori* infection in mice also induces autoantibodies that bind to parietal cells and that can be absorbed with synthetic Lewis antigen (34). Thus, in the murine system, *H. pylori* induces autoantibodies through mimicry. Moreover, high concentrations of circulating anti-Le^y MAbs may cause gastric damage (52). It was already known that *H. pylori* infection in humans also induces autoantibodies that recognize gastric parietal cells (27, 28, 52, 53), and in analogy with the *H. pylori* infection in mice, it was thought that those human autoantibodies also arose through mimicry. Indeed, in patient sera, high titers of antibodies to *H. pylori* LPS are found (6). However, the epitope-specificity of human anti-*H. pylori* LPS remains enigmatic; in an initial study, anti-Le^x antibodies were found in only a few patients' sera (6). However, in a larger survey comprising more than 100 patients, *H. pylori* infection was not found to induce anti-$Le^{x/y}$ antibodies in humans (19). In fact, anti-$Le^{x/y}$ antibodies occur naturally in sera from persons not infected by *H. pylori* (18). One exception might be nonsecretors (persons who do not express Le^b in gastric mucosa) where low affinity, *H. pylori*-associated anti-$Le^{x/y}$ antibodies were detected in serum (40). The question remains as to what epitopes of *H. pylori* LPS human antibodies are directed. Data have been presented that show that fucose is not part of the epitope recognized by human anti-*H. pylori* LPS antibodies, but the nature of this epitope remains elusive (75). Finally, antigastric autoantibodies present in sera of *H. pylori*-infected patients are directed to gastric parietal canaliculi, but absorption with *H. pylori* does not diminish autoantibody reactivity (26). This shows that the *H. pylori*-associated antigastric autoantibodies are not due to mimicry; further studies showed them to be directed to peptide epitopes of gastric H^+,K^+-ATPase (19). Thus, present data suggest that *H. pylori* $Le^{x/y}$ antigens do not induce autoantibodies in infected human patients. Humans are not per se unable to form anti-Le^x antibodies. Patients infected with *Schistosoma mansoni*, a tropical parasite that also expresses Le^x, develop serum antibodies to Le^x that are cytotoxic for Le^x (=CD15)-carrying leukocytes (54, 64). Why *H. pylori* does not induce serum anti-Le^x antibodies is not known. However, it cannot be excluded that *H. pylori* induces anti-$Le^{x/y}$ antibodies locally that bind directly

C. jejuni
H. pylori
Schistosoma mansoni

to gastric mucosal epitopes, so that they do not appear in serum.

Lewis Antigen Mimicry and Immune Evasion

H. pylori
H. mustelae

By analogy to the ABO blood group antigens, one might predict that a host that expresses Le^x would be expected to form anti-Le^y but not anti-Le^x antibodies. Hence, a Le^x-positive *H. pylori* strain that infects an Le^x-positive host would escape immune attack and be able to persist, while an Le^y-positive strain would not escape and would be eradicated. Experimental infection in rhesus monkeys confirms this concept: an *H. pylori* strain isolated from Le^y-positive animals (in gastric mucosa) expresses more Le^y than Le^x; the same strain expresses more Le^x than Le^y when isolated after colonization of Le^x positive animals (72). Thus, the expression of *H. pylori* $Le^{x/y}$ epitopes depends on the host. It is conceivable that in vivo outgrowth of Le^y-expressing *H. pylori* variants is favored because variants expressing Le^x are suppressed in Le^y-positive hosts that form anti-Le^x but not anti-Le^y antibodies. However, whether the two variants isolated are phase variants was not investigated, nor was it shown that the animals formed serum antibodies to $Le^{x/y}$. Studies in humans gave far less consistent results and, in two out of three studies, no correlation between the Lewis phenotypes of host and pathogen was found (36, 61, 74). In addition, strains expressing Le^x and strains expressing Le^y can be isolated from a single patient, an additional argument against adaptation based on Lewis antigens (73). Finally, selection and outgrowth of *H. pylori* $Le^{x/y}$ LPS variants would be driven by anti-$Le^{x/y}$ antibodies, and these are not found in infected patients (19). Despite these objections with regard to a role for Lewis antigen mimicry in immune evasion, it remains striking that ferrets, whose gastric epithelium is blood group A-positive, are colonized by a helicobacter species (*H. mustelae*) that also expresses blood group A (21, 48). It is also striking that *H. pylori* strains isolated from Chinese patients more often express Le^a or Le^b as compared to strains isolated in Western countries (76), while Chinese themselves also express the Le^{ab}-positive phenotype more often as compared to Caucasians.

H. pylori Lewis Antigens as Adhesins

H. pylori

Several host lectins are already known to interact with host Lewis antigens. For example, selectins bind to Le^x and, in particular, sLe^x (22, 42). Furthermore, several other C-type (calcium-dependent) lectins are known to interact specifically with mannose. Examples are mannose-binding protein, surfactant protein D, and macrophage mannose receptor (70). Mannose and fucose share the presence of two adjacent, equatorial OH– groups that are required for calcium-dependent interaction with this group of lectins. Hence, it is likely that fucosylated *H. pylori* LPS interact with C-type host lectins (see below).

H. pylori

Studies on the biological role of *H. pylori* Lewis antigens have largely taken place through insertional mutagenesis of LPS biosynthesis genes. The expression of $Le^{x/y}$ proved to be crucial for in vivo colonization of mice: the gene encoding β1,4 GalT was inactivated in strain SS-1

(expresses $Le^{x/y}$) (43). The mutant expresses a shorter LPS devoid of Lewis antigens and, in contrast to the parent strain, colonizes mice less well. However, the lack of colonization does not prove that Lewis antigens per se are essential: from other gram-negative pathogens it is known that shortening of LPS will lead to a decrease in virulence. Strains with a shorter LPS are simply more sensitive to the lytic action of serum or are more easily phagocytosed.

A double knockout was created in strain 4187E in which both α3-*fucT* genes were inactivated (4187E KO0379/0651) (see Table 3). This mutant expresses a long polylactosamine chain (i-antigen) and H type 1. The parent strain ($Le^{x/y}$ positive) colonizes mice well, but the mutant does not, which demonstrates that $Le^{x/y}$ antigens are essential for colonization (45). However, in another study, an α3-*fucT* double knockout colonized as well as its parent (16).

Recent data suggest that Le^x plays a role in adhesion. A MAb specific for *H. pylori* LPS inhibits adhesion of bacteria to gastric epithelial cells (56); this MAb is specific for Le^x (9). Further data on the role of Lewis x in adhesion were again obtained from knockout studies. Strains with a mutation in *galE* (HP0360, UDP-galactose-4-epimerase) yield a truncated LPS (24, 41) that lacks galactose (24). A strain knocked out in gene *rfbM* (HP0043, GDP-mannose pyrophosphorylase) yields a fucose-lacking LPS that expresses the i-antigen (24). *rfbM* is involved in biosynthesis of GDP-mannose, a precursor of GDP-fucose, which is the fucosyl donor of both α2- and α3-FucT. Both the *galE* and the *rfbM* mutant did not adhere to gastric sections, while the parent (strain NCTC 11637, $Le^{x/y}$ positive) adhered well (24). Infection studies with a *galE* mutant showed it to colonize less well than its parent (51a). In addition, synthetic Le^x coupled to 1 μm-sized polystyrene beads bound to human gastric epithelial cells (24). Clinical studies also suggest a role for $Le^{x/y}$ in adhesion; studies in gastritis patients demonstrated that *H. pylori* strains that expressed $Le^{x/y}$ strongly cause a higher colonization density than strains that express $Le^{x/y}$ weakly (36). In addition, a strong Lewis antigen expression of the infecting strain was associated with an increased influx of polymorphonuclear leukocytes (36). These data suggest that Le^x mediates colonization through adhesion, predict the existence of gastric Le^x-binding lectins, and suggest an association between adhesion and inflammation. Indeed, Le^x-binding lectins of 16 to 29 kDa (17) and 100 kDa (23) are found in the AGS gastric epithelial cell line; the identity of these proteins is unknown, but the presence of low molecular weight lectins (galectins) in the stomach has been reported (55). Other studies have shown that surfactant protein D, a C-type lectin belonging to the innate defense system and expressed in the stomach (30), is able to bind *H. pylori* LPS (25); it is unknown which moiety of the LPS is recognized. Thus, a role for LPS/$Le^{x/y}$ in adherence seems likely, but this role is not absolute. $Le^{x/y}$-negative mutants adhered as strongly as their $Le^{x/y}$-positive parents when the strain expresses the Le^b-binding lectin BabA and when the host expresses Le^b (14). In addition, $Le^{x/y}$-negative strains colonize human hosts well (58). Thus, an $Le^{x/y}$-lectin interaction may contribute to adhesion only for *H. pylori* strains that do not express BabA or for strains that colonize nonsecretors. Likewise, it is known that *H. pylori* can colonize mice, even when they do not express Le^b (34), the counter ligand of BabA (15, 38); colonization of mice might require the presence of Le^x-binding lectins in the gastric mucosa. Phase

HP0360
HP0043
H. pylori

variation might fulfill a biological role by allowing detachment of bacteria not expressing $Le^{x/y}$ and hence transmission to another host; subsequently, switch-back variants expressing $Le^{x/y}$ adhere and colonize a new host. Interestingly, variants that do not bind surfactant protein D have been isolated but colonization studies have not been performed with these strains (66).

Adhesion of *H. pylori* has clinical relevance: strains from ulcer patients more often express BabA compared to strains from gastritis patients (32). What is the link between adherence and development of host pathology? First of all, increased adherence may lead to an increased bacterial burden. Second, studies in mice show that increased adherence does not necessarily lead to increased colonization density but to a closer contact between bacteria and gastric epithelial cells (34). A more intimate contact enhances the crosstalk between microorganism and host and may lead to activation of transcription factor NF-κ and host signal transduction pathways (20). This induces interleukin-8 (IL-8) production and inflammation, and finally, ulceration. This sequence of events is in agreement with data that show that increased Le^{x} expression in *H. pylori* is associated with increased neutrophil infiltration (36), and that strains isolated from patients with ulcers express an increased number of Lewis antigens as compared to strains from dyspeptic patients (76).

H. pylori

In summary, the mechanisms of *H. pylori* LPS phase variation are known in detail; knowledge of the biological role of Lewis antigens and phase variation therein is in its infancy, but a role in adhesion seems likely.

H. pylori

References

1. Alm R. A., Ling L. S., Moir D. T., King B. L., Brown E. D., Doig P. C., Smith D. R., Noonan B., Guild B. C., deJonge B. L., Carmel G., Tummino P. J., Caruso A., Uria-Nickelsen M., Mills D. M., Ives C., Gibson R., Merberg D., Mills S. D., Jiang Q., Taylor D. E., Vovis G. F., Trust T. J. 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature* 397: 176-180. (PubMed)

2. Appelmelk B. J., Martin S. L., Monteiro M. A., Clayton C. A., McColm A. A., Zheng P. Y., Verboom T., Maaskant J. J., van den Eijnden D. H., Hokke C. H., Perry M. B., Vandenbroucke-Grauls C. M. J. E., Kusters J. G. 1999. Phase variation in *Helicobacter pylori* lipopolysaccharide due to changes in the lengths of poly(C) tracts in alpha 3-fucosyltransferase genes. *Infect. Immun.* 67: 5361-5366. (PubMed) (Full Text in PMC)

3. Appelmelk B. J., Martino M. C., Veenhof E., Monteiro M. A., Maaskant J. J., Negrini R., Lindh F., Del Giudice G., Vandenbroucke-Grauls C. M. J. E. 2000. Phase variation in the H type 1 and Lewis a epitopes of *Helicobacter pylori* lipopolysaccharide. *Infect. Immun.* 68: 5928-5932. (PubMed) (Full Text in PMC)

4. Appelmelk B. J., Negrini R., Moran A. P., Kuipers E. J. 1997. Molecular mimicry between *Helicobacter pylori* and the host. *Trends Microbiol.* 5: 70-73. (PubMed)

5. Appelmelk B. J., Shiberu B., Trinks C., Tapsi N., Zheng P. Y., Verboom T., Maaskant J., Hokke C. H., Schiphorst W. E., Blanchard D., Simoons-Smit I. M., van den Eijnden D. H., Vandenbroucke-Grauls C. M. 1998.

Phase variation in *Helicobacter pylori* lipopolysaccharide. *Infect. Immun.* 66: 70-76. (PubMed) (Full Text in PMC)

6. Appelmelk B. J., Simoons-Smit I., Negrini R., Moran A. P., Aspinall G. O., Forte J. G., De Vries T., Quan H., Verboom T., Maaskant J. J., Ghiara P., Kuipers E. J., Bloemena E., Tadema T. M., Townsend R. R., Tyagarajan K., Crothers, Jr J. M., Monteiro M. A., Savio A., de Graaff J. 1996. Potential role of molecular mimicry between *Helicobacter pylori* lipopolysaccharide and host Lewis blood group antigens in auto-immunity. *Infect. Immun.* 64: 2031-2040. (PubMed) (Full Text in PMC)

7. Appelmelk B. J., Vandenbroucke-Grauls C. M. 2000. *H. pylori* and Lewis antigens. *Gut* 47: 10-11. (PubMed)

8. Appelmelk B. J., Wirth H. P., Lansbergen R., Schilders I., Martin S. L., Verboom T., Vandenbroucke-Grauls C. M. J. E. 1999. *H. pylori* diversifies in the human host through lipopolysaccharide phase variation. *Gut* 45: A23-A24.

9. Appelmelk, B. J., and H. Yamaguchi. 2000. Unpublished data.

10. Aspinall G. O., Monteiro M. A. 1996. Lipopolysaccharides of *Helicobacter pylori* strains P466 and MO19: structures of the O antigen and core oligosaccharide regions. *Biochemistry* 35: 2498-2504. (PubMed)

11. Aspinall G. O., Monteiro M. A., Pang H., Walsh E. J., Moran A. P. 1996. Lipopolysaccharide of the *Helicobacter pylori* type strain NCTC 11637 (ATCC 43504): structure of the O antigen chain and core oligosaccharide regions. *Biochemistry* 35: 2489-2497. (PubMed)

12. Berg D. E., Hoffman P. S., Appelmelk B. J., Kusters J. G. 1997. The *Helicobacter pylori* genome sequence: genetic factors for long life in the gastric mucosa. *Trends Microbiol.* 5: 468-474. (PubMed)

13. Blixt O., van D., Norberg I. T., van den Eijnden D. H. 1999. High-level expression of the *Neisseria meningitidis* 1gtA gene in *Escherichia coli* and characterization of the encoded *N*-acetylglucosaminyltransferase as a useful catalyst in the synthesis of GlcNAc beta 1→3Gal and GalNAc beta 1→3Gal linkages. *Glycobiology* 9: 1061-1071. (PubMed)

14. Boren, T., and B. J. Appelmelk. 2000. Unpublished data.

15. Boren T., Falk P., Roth K. A., Larson G., Normark S. 1993. Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. *Science* 262: 1892-1895. (PubMed)

16. Camorlinga, M., E. El-Omar, and M. J. Blaser. 2000. *Helicobacter pylori* does not require Le ^{x} or Le ^{y} expression to colonize mice. *Abstr. 100th Gen. Meet. Am. Soc. Microbiol.* , abstr. D-268. American Society for Microbiology, Washington, D.C.

17. Campbell B. J., Rogerson K. A., Rhodes J. M. 1999. Adherence of *Helicobacter pylori* to human gastric epithelium mediated by an epithelial cell-surface Lewis(x) carbohydrate-binding protein. *Gastroenterology* 116: G0565.

18. Chmiela M., Jurkiewicz M., Wisniewska M., Czkwianianc E., Planeta-Malecka I., Rechcinski T., Rudnicka W. 1999. Anti-Lewis X IgM and IgG in *H. pylori* infections in children and adults. *Acta Microbiol. Pol.* 48: 277-281. (PubMed)

19. Claeys D., Faller G., Appelmelk B. J., Negrini R., Kirchner T. 1998. The gastric H+,K+-ATPase is a major autoantigen in chronic *Helicobacter pylori* gastritis with body mucosa atrophy. *Gastroenterology* 115: 340-347. (PubMed)

20. Covacci A., Rappuoli R. 2000. Tyrosine-phosphorylated bacterial proteins: Trojan horses for the host cell. *J. Exp. Med.* 191: 587-592. (PubMed)

21. Croinin O., Clyne M., Drumm B. 1998. Molecular mimicry of ferret gastric epithelial blood group antigen A by *Helicobacter mustelae*. *Gastroenterology* 114: 690-696. (PubMed)

22. Cummings, R. D. 1999. Selectins, p. 391–416. *In* A. Varki, R. D. Cummings, J. Esko, H. Freeze, G. Hart, and J. Marth (ed.), *Essentials of Glycobiology* . Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

23. Edwards, N. J. 2000. Data presented at the 4th Int. Workshop on Pathogenesis and Host Response in *Helicobacter* Infections, Helsingor, Denmark.

24. Edwards N. J., Monteiro M. A., Faller G., Walsh E. J., Moran A. P., Roberts I. S., High N. J. 2000. Lewis X structures in the O antigen side-chain promote adhesion of *Helicobacter pylori* to the gastric epithelium. *Mol. Microbiol.* 35: 1530-1539. (PubMed)

25. Eggleton P., Murray E., Dodds A., Worku M., Karim Q., Walker M., Ferris J., Moran A., Appelmelk B., Reid K., Thursz M. 1999. Surfactant protein D binding to *Helicobacter pylori* lipopolysaccharide. *Gut* 45: A35.

26. Faller G., Steininger H., Appelmelk B., Kirchner T. 1998. Evidence of novel pathogenic pathways for the formation of antigastric autoantibodies in *Helicobacter pylori* gastritis. *J. Clin. Pathol.* 51: 244-245. (PubMed) (Full Text in PMC)

27. Faller G., Steininger H., Eck M., Hensen J., Hann E. G., Kirchner T. 1996. Antigastric autoantibodies in *Helicobacter pylori* gastritis: prevalence, in-situ binding sites and clues for clinical relevance. *Virchows Arch.* 427: 483-486. (PubMed)

28. Faller G., Steininger H., Kranzlein J., Maul H., Kerkau T., Hensen J., Hahn E. G., Kirchner T. 1997. Antigastric autoantibodies in *Helicobacter pylori* infection: implications of histological and clinical parameters of gastritis. *Gut* 41: 619-623. (PubMed)

29. Farabaugh P. J. 1996. Programmed translational frameshifting. *Annu. Rev. Genet.* 30: 507-528. (PubMed)

30. Fisher J. H., Mason R. 1995. Expression of pulmonary surfactant D in rat gastric mucosa. *Am. J. Respir. Cell Mol. Biol.* 12: 13-18. (PubMed)

31. Ge Z. M., Chan N. W. C., Palcic M. M., Taylor D. E. 1997. Cloning and heterologous expression of an alpha 1,3-fucosyltransferase gene from the gastric pathogen *Helicobacter pylori. J. Biol. Chem.* 272: 21357-21363. (PubMed)

32. Gerhard M., Lehn N., Neumayer N., Boren T., Rad R., Schepp W., Miehlke S., Classen M., Prinz C. 1999. Clinical relevance of the *Helicobacter pylori* gene for blood-group antigen-binding adhesin. *Proc. Natl. Acad. Sci. USA* 96: 12778-12783. (PubMed) (Full Text in PMC)

33. Giardina, P. C., M. A. Apicella, B. Gibson, and A. Preston. 1999. Antigenic mimicry in *Neisseria* species, p. 55–65. *In* H. Brade, S. M. Opal, S. N. Vogel, and D. M. Morrison (ed.), *Endotoxin in Health and Disease* . Marcel Dekker, Inc., New York, N.Y.

34. Guruge J. L., Falk P. G., Lorenz R. G., Dans M., Wirth H. P., Blaser M. J., Berg D. E., Gordon J. I. 1998. Epithelial attachment alters the outcome of *Helicobacter pylori* infection. *Proc. Natl. Acad. Sci. USA* 95: 3925-3930. (PubMed) (Full Text in PMC)

35. Harvey H. A., Porat N., Campbell C. A., Jennings M., Gibson B. W., Phillips N. J., Apicella M. A., Blake M. S. 2000. Gonococcal lipooligosaccharide is a ligand for the asialoglyco-protein receptor on human sperm. *Mol. Microbiol.* 36: 1059-1070. (PubMed)

36. Heneghan M. A., McCarthy C. F., Moran A. P. 2000. Relationship of blood group determinants on *Helicobacter pylori* lipopolysaccharide with host lewis phenotype and inflammatory response. *Infect. Immun.* 68: 937-941. (PubMed) (Full Text in PMC)

37. Hood, D. W., and E. R. Moxon. 1999. Lipopolysaccharide phase variation in *Haemophilus influenzae* and *Neisseria* , p. 39–54. *In* H. Brade, S. M. Opal, S. N. Vogel, and D. M. Morrison (ed.), *Endotoxin in Health and Disease* . Marcel Dekker, Inc., New York, N.Y.

38. Ilver D., Arnqvist A., Ogren J., Frick I. M., Kersulyte D., Incecik E. T., Berg D. E., Covacci A., Engstrand L., Boren T. 1998. *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. *Science* 279: 373-377. (PubMed)

39. Knirel Y. A., Kocharova N. A., Hynes S. O., Widmalm G., Andersen L. P., Jansson P. E., Moran A. P. 1999. Structural studies on lipopolysaccharides of serologically non-typable strains of *Helicobacter pylori*, AF1 and 007, expressing Lewis antigenic determinants. *Eur. J. Biochem.* 266: 123-131. (PubMed)

40. Kurtenkov O., Klaamas K., Miljukhina L., Shljapnikova L., Ellamaa M., Bovin N., Wadstrom T. 1999. IgG antibodies to Lewis type 2 antigens in serum of *H. pylori*-infected and noninfected blood donors of different Lewis (a,b) blood-group phenotype. *FEMS Immunol. Med. Microbiol.* 24: 227-232. (PubMed)

41. Kwon D. H., Woo J. S., Perng C. L., Go M. F., Graham D. Y., El Zaatari F. A. 1998. The effect of *galE* gene inactivation on lipopolysaccharide profile of *Helicobacter pylori*. *Curr. Microbiol.* 37: 144-148. (PubMed)

42. Larsen E., Palabrica T., Sajer S., Gilbert G. E., Wagner D. D., Furie B. C., Furie B. 1990. PADGEM-dependent adhesion of platelets to monocytes and neutrophils is mediated by a lineage-specific carbohydrate, LNF III (CD15). *Cell* 63: 467-474. (PubMed)

43. Logan S. M., Conlan J. W., Monteiro M. A., Wakarchuk W. W., Altman E. 2000. Functional genomics of *Helicobacter pylori*: identification of a beta-1,4 galactosyltransferase and generation of mutants with altered lipopolysaccharide. *Mol. Microbiol.* 35: 1156-1167. (PubMed)

44. Martin S. L., Edbrooke M. R., Hodgman T. C., van den Eijnden D. H., Bird M. I. 1997. Lewis X biosynthesis in *Helicobacter pylori*. Molecular cloning of an alpha(1,3)-fucosyltransferase gene. *J. Biol. Chem.* 272: 21349-21356. (PubMed)

45. Martin S. L., McColm A. C., Appelmelk B. J. 2000. *H. pylori* adhesion and Lewis x. *Gastroenterology* 119: 1415-1416.

46. Monteiro M. A., Appelmelk B. J., Rasko D. A., Moran A. P., Hynes S. O., MacLean L. L., Chan K. H., Michael F. S., Logan S. M., O'Rourke J., Lee A., Taylor D. E., Perry M. B. 2000. Lipopolysaccharide structures of *Helicobacter pylori* genomic strains 26695 and J99, mouse model *H. pylori* Sydney strain, *H. pylori* P466 carrying sialyl Lewis X, and *H. pylori* UA915 expressing Lewis B classification of *H. pylori* lipopolysaccharides into glycotype families. *Eur. J. Biochem.* 267: 305-320. (PubMed)

47. Monteiro M. A., Chan K. H., Rasko D. A., Taylor D. E., Zheng P. Y., Appelmelk B. J., Wirth H. P., Yang M., Blaser M. J., Hynes S. O., Moran A. P., Perry M. B. 1998. Simultaneous expression of type 1 and type 2 Lewis blood group antigens by *Helicobacter pylori* lipopolysaccharides. Molecular mimicry between *H. pylori* lipopolysaccharides and human gastric epithelial cell surface glycoforms. *J. Biol. Chem.* 273: 11533-11543. (PubMed)

48. Monteiro M. A., Zheng P. Y., Appelmelk B. J., Perry M. B. 1997. The lipopolysaccharide of *Helicobacter mustelae* type strain ATCC 43772 expresses the monofucosyl A type 1 histo-blood group epitope. *FEMS Microbiol. Lett.* 154: 103-109. (PubMed)

49. Monteiro M. A., Zheng P. Y., Ho B., Yokata S., Amano K., Berg D. E., Chan K. H., MacLean L. L., Perry M. B. 2000. Expression of histo-blood group antigens by lipopolysaccharides of *Helicobacter pylori* strains from Asian hosts: the propensity to express type 1 blood group antigens. *Glycobiology* 10: 701-713. (PubMed)

50. Moran A. P., Appelmelk B. J., Aspinall G. O. 1996. Molecular mimicry of host structures by lipopolysaccharides of *Campylobacter* and *Helicobacter* spp: implications in pathogenesis. *J. Endotoxin Res.* 3: 521-531.

51. Moran A. P., Helander I. M., Kosunen T. U. 1992. Compositional analysis of *Helicobacter pylori* rough-form lipopolysaccharides. *J. Bacteriol.* 174: 1370-1377. (PubMed) (Full Text in PMC)

51a. Moran A. P., *et al.* 2000. The relationship between O-chain expression and colonization ability of *Helicobacter pylori* in a mouse model. *FEMS Immunol. Med. Microbiol.* 29: 263-270. (PubMed)

52. Negrini R., Lisato L., Zanella I., Cavazzini L., Gullini S., Villanacci V., Poiesi C., Albertini A., Ghielmi S. 1991. *Helicobacter pylori* infection induces antibodies cross-reacting with human gastric mucosa. *Gastroenterology* 101: 437-445. (PubMed)

53. Negrini R., Savio A., Poiesi C., Appelmelk B. J., Buffoli F., Paterlini A., Cesari P., Graffeo M., Vaira D., Franzin G. 1996. Antigenic mimicry between *Helicobacter pylori* and gastric mucosa in the pathogenesis of body atrophic gastritis. *Gastroenterology* 111: 655-665. (PubMed)

54. Nyame A. K., Pilcher J. B., Tsang V. C., Cummings R. D. 1996. *Schistosoma mansoni* infection in humans and primates induces cytolytic antibodies to surface Le(x) determinants on myeloid cells. *Exp. Parasitol.* 82: 191-200. (PubMed)

55. Oka T., Murakami S., Arata Y., Hirabayashi J., Kasai K., Wada Y., Futai M. 1999. Identification and cloning of rat galectin-2: expression is predominantly in epithelial cells of the stomach. *Arch. Biochem. Biophys.* 361: 195-201. (PubMed)

56. Osaki T., Yamaguchi H., Taguchi H., Fukuda M., Kawakami H., Hirano H., Watanabe S., Takagi A., Kamiya S. 1998. Establishment and characterization of a monoclonal antibody to inhibit adhesion of *Helicobacter pylori* to gastric epithelial cells. *J. Med. Microbiol.* 47: 505-512. (PubMed)

57. Rasko D. A., Wang G., Palcic M. M., Taylor D. E. 2000. Cloning and characterization of the alpha(1,3/4) fucosyltransferase of *Helicobacter pylori*. *J. Biol. Chem.* 275: 4988-4994. (PubMed)

58. Rasko D. A., Wilson T. J., Zopf D., Taylor D. E. 2000. Lewis antigen expression and stability in *Helicobacter pylori* isolated from serial gastric biopsies. *J. Infect. Dis.* 181: 1089-1095. (PubMed)

59. Saunders N. J., Peden J. F., Hood D. W., Moxon E. R. 1998. Simple sequence repeats in the *Helicobacter pylori* genome. *Mol. Microbiol.* 27: 1091-1098. (PubMed)

60. Simoons-Smit I. M., Appelmelk B. J., Verboom T., Negrini R., Penner J. L., Aspinall G. O., Moran A. P., Fei S. F., Shi B. S., Rudnica W., Savio A., de Graaff J. 1996. Typing of *Helicobacter pylori* with monoclonal antibodies against Lewis antigens in lipopolysaccharide. *J. Clin. Microbiol.* 34: 2196-2200. (PubMed) (Full Text

in PMC)

61. Taylor D. E., Rasko D. A., Sherburne R., Ho C., Jewell L. D. 1998. Lack of correlation between Lewis antigen expression by *Helicobacter pylori* and gastric epithelial cells in infected patients. *Gastroenterology* 115: 1113-1122. (PubMed)

62. Tomb J.-F., White O., Kerlavage A. R., Clayton R. A., Sutton G. G., Fleischmann R. D., Ketchum K. A., Klenk H. P., Gill S., Dougherty B. A., Nelson K., Quackenbush J., Zhou L., Kirkness E. F., Peterson S., Loftus B., Richardson D., Dodson R., Khalak H. G., Glodek A., McKenney K., Fitzegerald L. M., Lee N., Adams M. D., Venter J. C. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* 388: 539-547. (PubMed)

63. Tsuchihashi Z., Brown P. O. 1992. Sequence requirements for efficient translational frameshifting in the *Escherichia coli dnaX* gene and the role of an unstable interaction between tRNA(Lys) and an AAG lysine codon. *Genes Dev.* 6: 511-519. (PubMed)

64. van Dam G. J., Claas F. H., Yazdanbakhsh M., Kruize Y. C., van Keulen A. C., Ferreira S. T., Rotmans J. P., Deelder A. M. 1996. *Schistosoma mansoni* excretory circulating cathodic antigen shares Lewis-x epitopes with a human granulocyte surface antigen and evokes host antibodies mediating complement-dependent lysis of granulocytes. *Blood* 88: 4246-4251. (PubMed)

65. van Putten J. P. 1993. Phase variation of lipopolysaccharide directs interconversion of invasive and immuno-resistant phenotypes of *Neisseria gonorrhoeae*. *EMBO J.* 12: 4043-4051. (PubMed) (Full Text in PMC)

66. Walker M. M., Karim Q. N., Worku M., Murray E., Eggleton P., Reid K. B. M., Thursz M. R. 1999. Direct observation of kinetics and agglutination of *Helicobacter pylori* with the collectin, surfactant protein D. *Gut* 45: A41-A42.

67. Wang G., Boulton P. G., Chan N. W., Palcic M. M., Taylor D. E. 1999. Novel *Helicobacter pylori* alpha1,2-fucosyltransferase, a key enzyme in the synthesis of Lewis antigens. *Microbiology* 145: 3245-3253. (PubMed)

68. Wang G., Ge Z. M., Rasko D. A., Taylor D. E. 2000. Lewis antigens in *Helicobacter pylori*; biosynthesis and phase variation. *Mol. Microbiol.* 36: 1187-1196. (PubMed)

69. Wang G., Rasko D. A., Sherburne R., Taylor D. E. 1999. Molecular genetic basis for the variable expression of Lewis Y antigen in *Helicobacter pylori*: analysis of the alpha (1,2) fucosyltransferase gene. *Mol. Microbiol.* 31: 1265-1274. (PubMed)

70. Weis W. I., Taylor M. E., Drickamer K. 1998. The C-type lectin superfamily in the immune system. *Immunol. Rev.* 163: 19-34. (PubMed)

71. Wirth H. P., Karita M., Yang M., Blaser M. J. 1996. Expression of Lewis X and Y blood group antigens by

Helicobacter pylori strains is related to *cagA* status. *Gastroenterology* 110: A296. (PubMed)

72. Wirth H. P., Yang M., Dubois A., Berg D. E., Blaser M. J. 1998. Host Lewis phenotype-dependent selection of H-pylori Lewis expression in rhesus monkeys. *Gut* 43: A26.

73. Wirth H. P., Yang M., Peek, Jr R. M., Hook-Nikanne J., Fried M., Blaser M. J. 1999. Phenotypic diversity in Lewis expression of *Helicobacter pylori* isolates from the same host. *J. Lab. Clin. Med.* 133: 488-500. (PubMed)

74. Wirth H. P., Yang M., Peek, Jr R. M., Tham K. T., Blaser M. J. 1997. *Helicobacter pylori* Lewis expression is related to the host Lewis phenotype. *Gastroenterology* 113: 1091-1098. (PubMed)

75. Yokota S. I., Amano K. I., Shibata Y., Nakajima M., Suzuki M., Hayashi S., Fujii N., Yokochi T. 2000. Two distinct antigenic types of the polysaccharide chains of *Helicobacter pylori* lipopolysaccharides characterized by reactivity with sera from humans with natural infection. *Infect. Immun.* 68: 151-159. (PubMed) (Full Text in PMC)

76. Zheng P. Y., Hua J., Yeoh K. G., Ho B. 2000. Association of peptic ulcer with increased expression of Lewis antigens but not cagA, iceA, and vacA in *Helicobacter pylori* isolates in an Asian population. *Gut* 47: 18-22. (PubMed)

EXHIBIT 3

Am. J. Hum. Genet. 60:408–416, 1997

Homopolymeric Tract Heteroplasmy in mtDNA from Tissues and Single Oocytes: Support for a Genetic Bottleneck

D. R. Marchington,[1] G. M. Hartshorne,[2,*] D. Barlow,[2] and J. Poulton[1]

[1]Department of Paediatrics and [2]Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Oxford

Summary

While mtDNA polymorphisms at single base positions are common, the overwhelming majority of the mitochondrial genomes within a single individual are usually identical. When there is a point-mutation difference between a mother and her offspring, there may be a complete switching of mtDNA type within a single generation. It is generally assumed that there is a genetic bottleneck whereby a single or small number of founder mtDNA(s) populate the organism, but it is not known at which stages the restriction/amplification of mtDNA subtype(s) occur, and this uncertainty impedes antenatal diagnosis for mtDNA disorders. Length polymorphisms in homopolymeric tracts have been demonstrated in the large noncoding region of mtDNA. We have developed a new method, T-PCR (trimmed PCR), to quantitate heteroplasmy for two of these tracts (D310 and D16189). D310 variation is sufficient to indicate clonal origins of tissues and single oocytes. Tissues from normal individuals often possessed more than one length variant (heteroplasmy). However, there was no difference in the pattern of the length variants between somatic tissues in any control individual when bulk samples were taken. Oocytes from normal women undergoing in vitro fertilization were frequently heteroplasmic for length variants, and in two cases the modal length of the D310 tract differed in individual oocytes from the same woman. These data suggest that a restriction/amplification event, which we attribute to clonal expansion of founder mtDNA(s), has occurred by the time oocytes are mature, although further segregation may occur at a later stage. In contrast to controls, the length distribution of the D310 tract varied between tissues in a patient with heteroplasmic mtDNA rearrangements, suggesting that these mutants influence segregation. These findings have important implications for the genetic counselling of patients with pathogenic mtDNA mutations.

Received March 5, 1996; accepted for publication November 5, 1996.

Address for correspondence and reprints: Dr. Joanna Poulton, Department of Paediatrics, Level 4, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom. E-mail: joanna.poulton@paediatrics.ox.ac.uk

*Present affiliation: Department Biological Sciences, University of Warwick, Coventry.

0002-9297/97/6002-0020$02.00

Introduction

Mutations in human mtDNA accumulate ~10 times faster than in nuclear DNA so that mtDNA polymorphisms are common. There are thousands of copies of mtDNA in a single cell, and yet most of the mitochondrial genomes in a single human control are identical (homoplasmy) (Monnat and Loeb 1985). In contrast, heteroplasmy (two or more populations of mtDNA in a single individual) is common in patients with mtDNA diseases. The reason for this is unknown, but it may be that in some cases homoplasmy for mutant mtDNAs is lethal.

Despite the fact that there are thousands of mtDNAs in a cell, when there is a neutral point mutation difference between a mother and her offspring, there may be complete switching of mtDNA genotype in a single generation, as has been demonstrated in Holstein cows (Koehler et al. 1991). If this switching were caused by random segregation or drift, heteroplasmy for polymorphic point mutations should be relatively common. In practice, it has been reported only rarely. Alternatively, the state of homoplasmy may be maintained by a restriction/amplification event, or "bottleneck," whereby a small number of mtDNA molecules ultimately populate the organism. By analyzing instances where segregation is not complete, investigators have estimated the number of mtDNAs that may be founders of the adult genotype. Estimates range from 1–6 (Hauswirth and Laipis 1985) and 20–100 in cows (Ashley et al. 1989) to 370–740 segregating units in *Drosophila* (Solignac et al. 1987). The segregating unit could represent the mtDNA complement of a single mitochondrion or subgroup of mitochondria in humans. It is not known at which stage this restriction/amplification of one mtDNA subtype might occur, nor is its molecular basis known. However, there is a 50-fold increase in the number of mtDNAs per cell, from 4,000 to 200,000, during oogenesis. Daughter cells could be clonal, with respect to mtDNA, if they originate from regions of cytoplasm within the oocyte where clones derived from founder mtDNAs remain in clusters (Hauswirth and Laipis 1985).

Length polymorphisms have been demonstrated in homopolymeric tracts within the large noncoding region of mtDNA (Hauswirth and Clayton 1985; Bendall and Sykes 1995). Heteroplasmy of this length variation in

homopolymeric tracts has been recognized because of blurring of bands on a sequencing gel after the tract. That this was not a sequencing artifact was demonstrated by cloning and sequencing of individual clones, at which point the sequence becomes readable (Bendall and Sykes 1995; Marchington et al. 1996). In order to identify heteroplasmic variants that might be used as markers for different mtDNA clones within the germline and somatic cells of normal individuals, we investigated the variation in two of these homopolymeric tracts. Minisatellite repeats with extremely high mutation rates have been used to investigate nuclear variation in germ-line and somatic cells (Monckton et al. 1994), while point mutations, which evolve more slowly, are suitable for more deeply rooted relationships. By analogy, we required a mtDNA polymorphism with a high rate of variation for these studies. We have developed a new method, T-PCR (trimmed PCR) to quantitate heteroplasmy for two mtDNA homopolymeric tracts (D310 and D16189) in tissues and oocytes (table 1). We found that D310 was sufficiently stable for length variants to cosegregate with clonal mtDNA populations but that D16189 was not suitable. We have used an analysis of length variation of D310 to investigate the segregation of mtDNA molecules within single oocytes of humans and mice.

Subjects and Methods

Patient Material

Ethical approval was obtained from the Central Oxford Research Ethics Committee. Oocytes that had failed to fertilize 2 d after insemination in vitro were donated for research by five women undergoing in vitro fertilization (IVF). Three to eight oocytes from each woman were available and used in this study. Oocytes were incubated in individual petri dishes. Oocytes were removed from the dishes and washed in sterile PBS. Adherent sperm and the zona pellucida were removed by brief exposure to PBS adjusted to pH 2.6, a modification of the acidic Tyrode solution method. The oocytes were placed in fresh PBS and checked for the absence of sperm. Thus, oocyte mtDNA was in a vast excess over any residual traces of paternal mtDNA derived from any adherent sperm.

Two to five postmortem tissues samples were obtained from five subjects who died from nonneurological causes (controls). Patient 1 had Kearns-Sayre syndrome (KSS) and rearranged mtDNA, comprising duplications, deletions, and deletion dimers, in addition to normal mtDNA as described by Poulton et al. (1995). Nine different tissues were available. Length variation in D16189 was previously reported in patient 2 (Marchington et al. 1996), who had a T:A→G:C transition at bp 3243, which is a pathogenic mutation associated with MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes).

Mice

Oocytes were dissected from the ovaries of a C57BL/6 mouse. The ovaries were washed and placed in sterile PBS. The tissue was dissected in PBS under a dissecting microscope and the oocytes released were removed and placed in fresh PBS. The oocytes were examined by light microscopy, and any that were damaged or had adherent cells were discarded. Nineteen intact oocytes were obtained.

Table 1

T-PCR of the Variable Tracts

	Tract		
Tract Name	D16189	D310	Mouse D310 Homologue
Wild-type sequence	CCCCCTCCCC	CCCCCCCTCCCCC	CCCCCCACCCCC
Normal situation	Homoplasmic	Heteroplasmic	Heteroplasmic
Forward primer (bp)	16161–16183	266–285	16068–16087
Forward primer sequence	TAAAAACCCAATCCACATCAAA	TTCCACACAGACATCATAAC	CCAAATTTTAACTCTCCAAA
Reverse primer (bp)	325–344	580–599	10–31
Reverse primer sequence (biotinylated)	AGATGTGTTTAAGTGCTGT	TTGAGGAGGTAAGCTACATA	GTGCTTTGCTTTGTTATTAAGC
Polymorphism giving rise to length variation	15% Caucasians	Not applicable	Not applicable
Restriction enzyme	*RsaI*	*HaeIII*	*DdeI*
Recognition/cutting site	GT→AC 16209	GG→CC 323	C→TAAG 16127
Size of PCR product (bp)	752	333	258
Size of fragment released (bp)	48+/− tract-length variation	57+/− tract-length variation	59+/− tract-length variation

T-PCR

We investigated two homopolymeric tracts within the large noncoding region of mtDNA near bp 16189 and 310 (henceforth D16189 and the D310 tract, respectively; see table 1). In most individuals, the D16189 tract consists of a run of 10 C:Gs, with a T:A at bp 16189. However, in ~15% of Caucasians, there is a T:A→C:G mutation at bp 16189. The homopolymeric tract resulting from this mutation may vary from 8 to 14 bp in length. The D310 tract consists of a run of 12–18 C:Gs, with a T:A near the middle at bp 310. To quantitate the proportions of each length variant, we have developed a PCR-based method. Short PCR products of ~40–50 bp were end-labeled with ^{32}P and separated on sequencing gels, and the distribution of the length variants was quantitated by phosphorimager analysis and autoradiography. This simple procedure may give rise to artifactual length variants because *Taq* polymerase can introduce a 1-bp overhang at the 3′ end in PCR products, and primers are usually contaminated with low levels of incomplete oligonucleotides. Furthermore, single-stranded DNA and its complementary strand may migrate to different positions on a denaturing gel. Primer dimers also run at 40–50 bp. We developed T-PCR, to avoid these confounding problems, and figure 1*A* shows a scheme for T-PCR of the D310 tract. PCR products of several hundred base pairs, encompassing the tract of interest, were generated (see table 1). A 5′-primer (PAGE purified to a single length for incorporation into the final product), adjacent to the tract, was used with a biotinylated antisense primer (which blocks end-labeling of that strand). PCR products were immobilized by binding to streptavidin-coated beads (Dynal). The immobilized products were washed, end-labeled (on the nonbiotinylated strand only) with γ-^{32}P dATP by polynucleotide kinase, washed, and incubated with a restriction enzyme that cuts just downstream of the homopolymeric tract of interest. The size of the labeled product cleaved from the beads reflects the length of the homopolymeric tract and is used to quantitate length variants. The 3′ end with heterogeneous overhang and possible contaminants, such as primer dimers and products that result from false priming, remains attached to the beads. The short labeled products were free from unincorporated label and were heated at 75°C for 2 min with a denaturing buffer before being run on an 8% sequencing gel at 75 W for 2–3 h. Gels were dried under vacuum and exposed to X-ray film or phosphorimager plates.

In order to ensure that any apparent heteroplasmic length variation was not a PCR artifact, PCR products containing the D310 tract were cloned into M13 (the host was DH5α, which is recA$^-$). Clones were reamplified by PCR, and these PCR products were cloned. Fifty of these clones were sequenced, and in all cases the track length was identical. Cloned DNA was amplified by T-PCR, and at no time was more than a single length obtained (results not shown).

Standard PCR conditions were used (Marchington et al. 1996) for 40 cycles, in all cases, and the starting quantities of template were ~10^4 and 10^8 copies of mtDNA per reaction for eggs and tissues, respectively. We demonstrated that the distribution of length variants was not influenced by low copy number (which might result in "allelic dropout") by diluting tissue samples down to a level below the limit of detection of any product ($<10^2$). We confirmed that T-PCR was quantitative by mixing, in various proportions, DNA samples (from tissues or cloned DNA as above) that were known by sequence analysis to have D310 tracts of different length and analyzing them by T-PCR (figs. 1*B* and 1*C*). Figure 1*B* shows autoradiographs obtained by mixing DNA from two different individuals' muscle tissue. Figure 1*C* demonstrates that, after quantitation by a phosphorimager, the proportion of the signal attributable to each sample varied with their proportion in the sample.

Results

The variation rate of the D310 and D16189 tracts were compared in cybrid lines (courtesy of Dr. I. J. Holt) derived from a patient with both the 3243 mutation associated with MELAS and the 16189 T:A→C:G polymorphism (Marchington et al. 1996). The cybrids were derived by fusing cytoplasts from patient 2's myoblasts with a mtDNA-free cell line. Figure 2 illustrates length variation in D310 and D16189 tracts in muscle tissue and muscle cell cybrids from patient 2 containing different proportions of 3243 G:C mutation In the D310 tract, the 0% mutant sample (lane 2) clearly segregates with a different major length variant (length variant 3) relative to the samples containing high levels of the 3243 G:C mutation (lanes 1, 3, and 4), which segregate with length variant 2. However, D16189 tract variants displayed the same distribution, irrespective of the proportions of 3243 G:C mutation, demonstrating that the same proportion of 3243 mutation was present in each length variant. Thus, in this culture system, specific D310 variants cosegregated with the 3243 mutant, but D16189 variants did not.

Control individuals were investigated for heteroplasmic length variation in the D310 tract in 2–5 different tissues. Figure 3 shows that the length of the D310 tract can vary between individuals. Subjects 2, 3, and 5 were homoplasmic, with a single length variant in the D310 tract. In subjects 1 and 4, there were low levels of a shorter-length variant. In subject 1, this varied from 1% to 4% and in subject 4 from 4% to 5%. Thus, although there may be some degree of heteroplasmy within the five normal individuals, a single modal length accounted for $\geq$95% of the length variants within an individual.

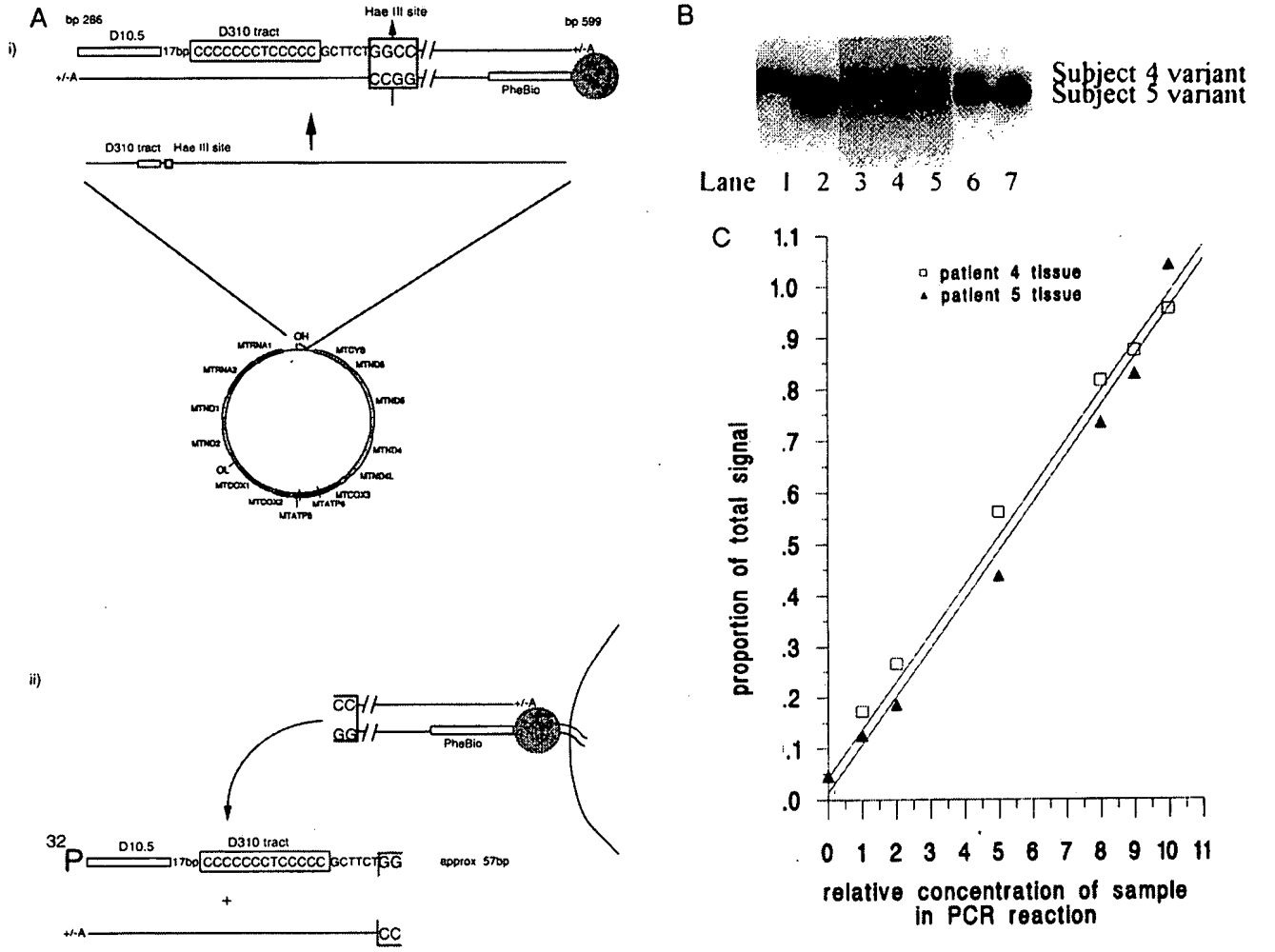
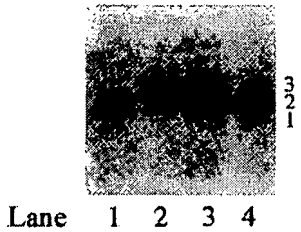
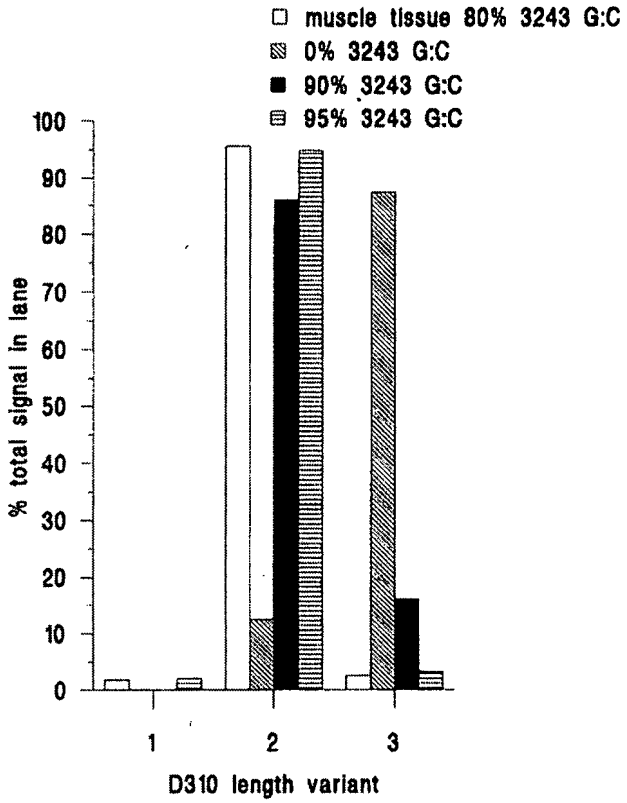


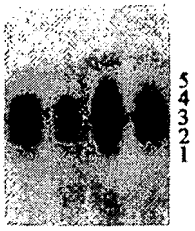
Figure 1 D310 tract T-PCR. *A*, Scheme of T-PCR for the D310 tract. (i) The 5′ primer (D10.5) was selected to be slightly upstream of the D310 tract, and a *Hae*III site was identified just downstream of the tract. The 3′ biotinylated primer (PheBio) was positioned several hundred base pairs downstream of the tract to provide a long "handle" and to ensure separation of products after digestion with the restriction enzyme. (ii) The PCR products were immobilized on streptavidin-coated beads, and the strand containing the PAGE-purified D10.5 primer was end-labeled with ^{32}P (the biotin blocks labeling of the other strand). Incubation of the beads with the restriction enzyme *Hae*III releases a labeled product of ~57 bp (whose exact size reflects the length of the homopolymeric tract and which was used to quantitate length variation therein). The other product of the digestion (276 bp) remains attached to the beads. *B*, Muscle DNA from two subjects (4 and 5) with different length D310 tracts, which were mixed in various proportions (v/v) and samples subjected to T-PCR as described in Subjects and Methods. Mixings are as a ratio of patient 4:patient 5: Lane 1, 10:0; Lane 2, 0:10; Lane 3, 9:1; Lane 4, 8:2; Lane 5, 5:5; Lane 6, 2:8; and Lane 7, 1:9. *C*, T-PCR products, which were quantitated by phosphorimager. For each band, the proportion of total signal in the lane was calculated and plotted as a function of the concentration of the sample in the PCR reaction. This demonstrates that T-PCR can be used to quantitate accurately length variants in the D310 tract. Muscle from subject 4 was heteroplasmic for length variation and contained 4% of the shorter variant found in subject 5; this is reflected in the displacement of the assay point with 0% subject 5 DNA by this amount.

Single oocytes were examined for evidence of mtDNA heteroplasmy. Figure 4 illustrates D310 tract variation in single oocytes from a single mouse ovary and in single human oocytes from patients attending the Oxford IVF Unit. Almost all oocytes were heteroplasmic. Figure 4*A* shows that, in the mouse, ~25% of oocytes differed from the mean pattern, and there were two cases, lanes 1 and 8, where the major length variant was also different. In human oocytes (fig. 4*B*), the tract variation pattern was the same in all oocytes from each of three of five donors (subjects 7–9 in fig. 4*B*). In the other two subjects (6 and 10) there are at least two different major length variants.

Figure 5 shows that there was a greater degree of heteroplasmy and variation of the D310 tract between tissues in a patient with mitochondrial disease than in controls, and this was confirmed by phosphorimager analysis. There were two length variants in each tissue, but the proportions varied. The extremes of variation were between spleen (lane 2) and liver (lane 7). In spleen,







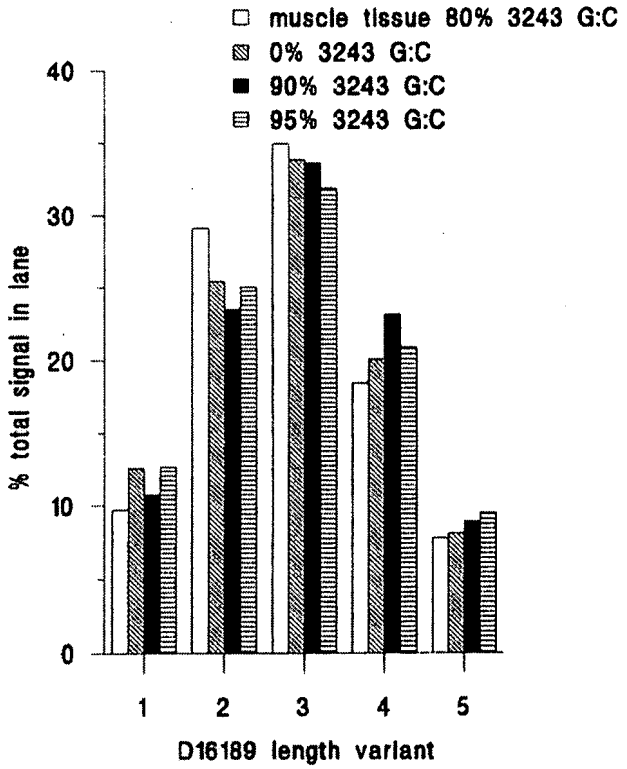


Figure 2 Comparison of length variation in different homopolymeric tracts and cloned muscle cell hybrids from patient 2, who is heteroplasmic for a pathological point mutation at bp 3243. Length variation was assessed in D310 (*upper panel*) and D16189 (*lower panel*) tracts by T-PCR. Lane 1, muscle (80% 3243 G:C). Lanes 2–4, muscle-cell hybrids containing 0%, 90%, and 95% 3243 G:C, respectively. The graphs show phosphorimager analysis of the T-PCR products; the length variants are numbered as on the autoradiographs. In the D310 tract, length variant 2 tends to segregate with the 3243 mutation, while length variant 3 tends to segregate with wild type. However, in the D16189 tract, length variants display the same distribution, irrespective of the proportions of 3243 G:C mutant.

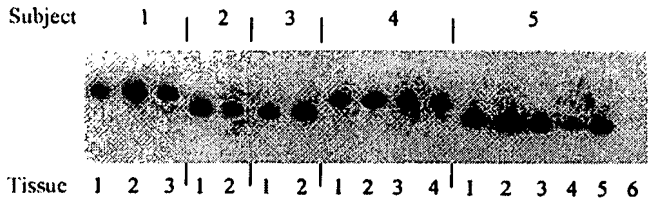


Figure 3 D310 length variation in tissues from controls. The autoradiographs show T-PCR of DNA from tissues of five subjects as described in Subjects and Methods. The tissues were (from *left* to *right*) subject 1, (1) muscle, (2) heart, and (3) liver; subject 2, (1) heart and (2) liver; subject 3, (1) muscle and (2) heart; subject 4, (1) muscle, (2) heart, (3) liver, and (4) kidney; subject 5, (1) heart, (2) liver, (3) kidney, (4) brain, and (5) pectoralis. Lane 6, subject 5, a water control. This demonstrates that bulk tissue samples may be heteroplasmic but that the modal length is identical in all tissues studied in an individual.

5% of the signal was found in the lower band, whereas in liver the value was 40%.

Discussion

We have used length variation in homopolymeric tracts around bp 310 and 16189 of mtDNA to investigate heteroplasmy in normal controls and patients with mitochondrial disease. We suggest that it is legitimate to use D310 but not D16189 as a marker of clonal origin of mtDNA. Figure 2 shows that, in contrast with the D310 tract, no particular D16189 variants cosegregated with the 3243 G:C mtDNAs in the MELAS patient. This suggests that, in cell culture, length variants were generated more rapidly in D16189 than in the D310 tract. Therefore, rapid generation of D16189 variants would conceal segregation of mtDNA clones. It has been shown that an identical distribution of D16189 variants can be passed down a pedigree (Bendall and Sykes 1995; Marchington et al. 1996), and this has been used as evidence against a narrow mtDNA bottleneck. Conversely, a bottleneck could be concealed by length variation generated during oogenesis and/or embryogenesis in these maternal lineages, as occurred in our MELAS patient cell lines. Therefore, we suggest that the D310, but not the D16189, tract may be a useful marker of mtDNA clonality. In both cases, length variation is presumably caused by replication slippage (Hauswirth et al. 1984), but it is not clear why there is a difference in the rate of generation of length variants between the two tracts. While the presence of a T:A base pair interrupting the C:G tract appears to ensure stability in the wild-type D16189 tract, this interruption is not sufficient to stabilize the D310 tract. Once the T:A base pair in the D16189 tract is lost, this tract may become markedly less stable than the D310 tract. However, the presence of this T:A base pair in the D16189 tract cannot be the only determinant of stability, because a continuous C:G tract is not always associated with length variation (Marchington et al. 1996).

We have confirmed that the D310 tract may vary in modal length between individuals (Horai and Hayasaka 1990). Other authors have shown that heteroplasmic

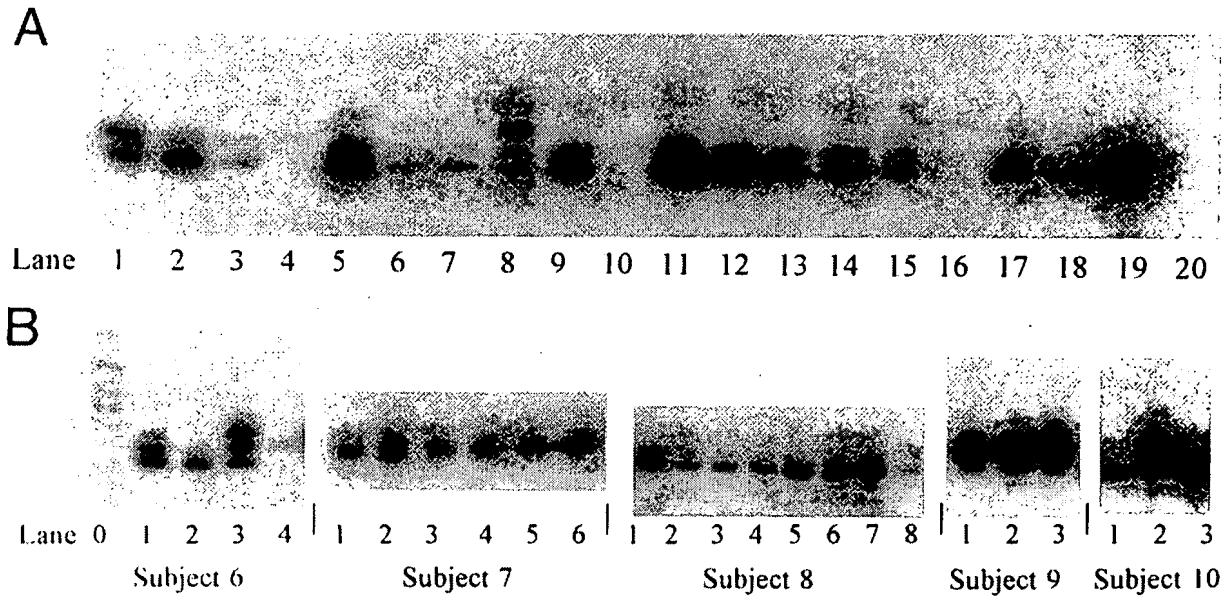


Figure 4 D310 tract variation in single oocytes. *A*, Mouse equivalent of the D310 tract, investigated by T-PCR in single oocytes dissected from a mouse (C57BL/6) ovary, lanes 1–19. Lane 20 is a water control. *B*, Autoradiographs, which show D310 tract variation, assessed by T-PCR, in single oocytes from the Oxford IVF Unit (subjects 6–10). Lane 0 in the subject 6 panel is a 1-bp sequencing ladder. This demonstrates that the modal length of the D310 tract may vary between oocytes from a single control.

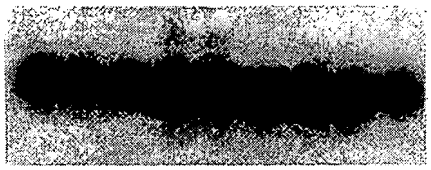


Figure 5 D310 length variation in a patient with KSS. The autoradiographs show T-PCR of DNA from tissues of patient 1. The tissues were (1) pituitary, (2) spleen, (3) muscle, (4) heart, (5) brain, (6) pancreas, (7) liver, (8) ovary, and (9) pectoralis. This demonstrates that the mean length of the D310 tract may vary between tissues in this patient.

substitutions and single-base-pair insertion/deletion mutations occur at a high frequency in the D310 tract (Jazin et al., in press). We have demonstrated that normal individuals may be heteroplasmic for D310 length variants. While heteroplasmy is frequently reported in mitochondrial disease, presumably because homoplasmy for many of the underlying pathogenic mutations would be lethal in many instances, heteroplasmy for mitochondrial polymorphisms has been reported only occasionally (Howell et al. 1992; Jazin et al., in press).

In normal individuals, there may be a degree of heteroplasmy in the D310 tract length in the tissues investigated. Moreover, the distribution of length variants of this tract appears to be identical among different tissues within each individual (fig. 3). This suggests that the mtDNA in each bulk tissue, within a normal individual, arises from the same founder mtDNA(s). The small number of additional length variants, which may be generated by replication slippage during expansion of the mtDNA population, may be masked by the excess of the modal length variant.

We found more length variation in single oocytes from humans and mice than in bulk samples from tissues. Although the human oocytes had been in the presence of sperm, it is highly unlikely that they could have been contaminated by significant quantities of sperm mtDNA. A single sperm contains ~50 mtDNAs (Hecht et al. 1984). Any sperm mtDNA that had penetrated the oocyte would be diluted by a factor of 2,000. It is conceivable that some sperm mtDNA might have been solubilized following removal of the zona in acid PBS and been transferred with a small volume of PBS in subsequent washes. If so, it would have been diluted in excess of 2,500-fold in the PBS and a further 2,000-fold by the maternal mtDNA. It is highly unlikely that contamination on this scale could result in a shift in the modal length of the PCR product. In addition, similar results were obtained with mouse oocytes that had never been exposed to sperm. Therefore, any mtDNA from nonoocyte ovarian tissue would have been diluted out ≥10,000-fold as with the human oocytes–washing/transfer procedure, and tract variants most likely arose from the oocyte mtDNA.

The presence of different modal lengths of the D310 tract in individual oocytes from "normal" women suggests that a restriction/amplification event must have occurred between conception and maturation of oocytes. For instance, in subject 10 in figure 4*B*, the difference in modal length between the oocyte in lane 1 (or lane 3) as opposed to the oocyte in lane 2 suggests expansion of different founder mtDNAs in the two oocytes. This difference could have arisen by clonal expansion of a single or a few mtDNAs within each oocyte, by segregation with drift, or by a combination of both. We suggest that clonal expansion of founder mtDNAs may make the major contribution to this difference, since there is a 50-fold increase in the number of mtDNAs during oogenesis in cows, from ~4,000 in oogonia to ~200,000 in mature oocytes, coincident with a reduction in the number of mtDNAs per mitochondrion (Hauswirth and Laipis 1985). The smaller the number of segregating units for mtDNA in the germ line, the more likely it is that mtDNA in the progeny will be clonal, provided that all the mtDNA molecules in one segregating unit are identical. However, the high mutation rate of mtDNA, particularly in the large noncoding region that includes the D310 tract, may allow a degree of length variation in the tract by the time the organism is mature. As with the bulk tissue samples, any minority length variants may be barely detectable, unless cells that are clonal with respect to mtDNA, such as in individual oocytes, are selected. The number of founder mtDNAs cannot be estimated where multiple D310 length variants were detectable because new lengths may be generated during the course of clonal proliferation. However, a difference in the modal tract length in two samples from the same individual is highly suggestive of different founder mtDNAs. The presence of different modal lengths of the D310 tract in individual oocytes in a proportion of "normal" women is the first direct evidence suggesting that restriction/amplification of founder mtDNA(s) has occurred by the time normal human oocytes are mature. Such clonal expansion could underlie the rapid switching of apparently neutral polymorphisms between generations. Information about the timing of this restriction/amplification event is an essential prerequisite if prenatal diagnosis of mtDNA disorders is to become feasible. Provided that the major component of this rapid switching occurs in the female germ line, quantitation of the level of mutant mtDNA in chorionic villus may be a good reflection of the level in the whole embryo.

Because the oocyte donors were attending an infertility clinic, it is not certain that these oocytes would have been viable and hence represent the normal situation

accurately. However, as before, similar results were obtained on oocytes from a normal virgin mouse (fig. 4*A*). The oocytes have been through some sort of restriction/amplification event, whether or not viability is low. This is also consistent with unpublished findings in a mouse model of heteroplasmy: the proportions of two populations of mtDNAs in mature oocytes reflected those in the newborn offspring, suggesting that a restriction/amplification event or "bottleneck" precedes the final maturation of oocytes (Jenuth et al. 1996). Rapid segregation could contribute to this switching of mtDNA populations in pathogenic mtDNA mutations, which may occur in vitro (Yoneda et al. 1992; Dunbar et al. 1995). We investigated two patients with pathogenic mtDNA mutations, both of whom were heteroplasmic for wild-type and mutant mtDNA. Figure 5 shows a greater degree of length variation between tissues in a patient with a heteroplasmic rearrangement of mtDNA (Poulton et al. 1995) than in controls (fig. 3). This may indicate that additional factors (such as the effect of impaired mitochondrial function on cell growth or faster replication of mutant than wild-type mtDNAs in certain tissues) may influence transmission of mtDNA in disease. Segregation of length variants in this case may depend on cosegregation with members of the different populations of mutant mtDNA. Furthermore, it is likely that further changes in the proportion of mtDNA mutant occur during subsequent clonal expansion and after birth (Poulton and Morten 1993; Matthews et al. 1994). This does not conflict with earlier views on the bottleneck. Segregation studies in mtDNA disease have suggested that several mtDNAs, rather than a single mtDNA, generally populate the progeny of affected females. The segregating unit may be a mitochondrion that contains several mtDNAs and hence is potentially heteroplasmic. Without knowing whether individual mitochondria can be heteroplasmic, attempts to calculate the number of segregating units may be flawed. However, rapid switching between different mtDNA types occasionally occurs, suggesting that a segregating unit may be homoplasmic for wild type. Attempts to calculate the number of segregating units in normal individuals are few. Howell et al. (1992) found heteroplasmy at a polymorphic site (bp 14,560) in a family with Leber hereditary optic neuropathy due to a pathogenic mutation at bp 3460 (which was homoplasmic). At bp 14,560, there was a silent G:C→A:T transition at the third base position in a codon specifying valine. The proportion of mutant ranged from 22% to 66% in the progeny. This failure of wild-type and mutant mtDNA to segregate to homoplasmy suggests again that mtDNAs that are wild type and mutant at bp 14,560 could be grouped together in segregating units. It could perhaps be maintained by large numbers of mtDNAs per mitochondrion or conceivably by aggregation of mtDNAs in multimeric forms such as catenates. For instance, mutant resolvases in yeast may result in biased transmission by aggregation of unresolved multimeric mtDNAs into large segregating units (Lockson et al. 1995).

Conclusion

These data support the concept of a mtDNA bottleneck whereby a few founder molecules populate the organism by demonstrating more mtDNA variation between oocytes than between tissues within single individuals. We suggest that a bottleneck occurs in oogenesis before the formation of mature oocytes. If this is the major determinant of mtDNA segregation between generations, antenatal diagnosis for mtDNA diseases may become feasible. However, it is likely there may be further segregation during development (Matthews et al. 1994).

Acknowledgments

Financial support was from the Wellcome Trust. We would like to thank the patients and their physicians for their help and cooperation and especially Dr. Ian Holt for the cybrid cell lines on the MELAS patient. We would like to thank Prof. N. Howell and Drs. K. Morten, G. Brown, and B. Sykes for helpful discussions, Mary Deadman for technical help, and Prof. E. R. Moxon for his continuing support and encouragement.

References

Ashley C, Laipis P, Hauswirth W (1989) Rapid sequestration of heteroplasmic bovine mitochondria. Nucleic Acids Res 17: 7325–7231

Bendall KE, Sykes BC (1995) Length heteroplasmy in the first hypervariable segment of the human mtDNA control region. Am J Hum Genet 57:248–256

Dunbar D, Moonie P, Jacobs H, Holt I (1995) Different cellular backgrounds confer a marked advantage to either mutant or wild-type mitochondrial genomes. Proc Natl Acad Sci USA 92:6562–6566

Hauswirth W, Laipis P (1985) Transmission genetics of mammalian mitochondria: a molecular model and experimental evidence. In: Quagliarello E, Slater EC, Palmierie F, Saccone G, Kroon M (eds) Achievements and perspectives of mitochondrial research. Vol 2: Biogenesis. Elsevier Biomedical, Amsterdam, pp. 49–59

Hauswirth W, Walle MVD, Laipis P, Olivo P (1984) Heterogeneous mitochondrial DNA D-loop sequences in bovine tissue+. Cell 37:1001–1007

Hauswirth WW Clayton DA (1985) Length heterogeneity of a conserved displacement-loop sequence in human mitochondrial DNA. Nucleic Acids Res 13:8093–8104

Hecht NB, Liem H, Kleene KC, Distel RJ, Ho SM (1984) Maternal inheritance of the mouse mitochondrial genome

is not mediated by a loss or gross alteration of the paternal mitochondrial DNA or by methylation of the oocyte mitochondrial DNA. Dev Biol 102:452–461

Horai S, Hayasaka K (1990) Intraspecific nucleotide sequence differences in the major noncoding region of human mitochondrial DNA. Am J Hum Genet 46:828–842

Howell N, Halvorson S, Kubacka I, McCullough DA, Bindoff LA, Turnbull DM (1992) Mitochondrial gene segregation in mammals: is the bottleneck always narrow? Hum Genet 90:117–120

Jazin EE, Cavelier L, Eriksson I, Oreland L, Gyllensten U. Mitochondrial DNA sequence heteroplasmy and mutation load in human brain. Proc Natl Acad Sci USA (in press)

Jenuth JP, Peterson A, Fu K, Shoubridge EA (1996) Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA. Nat Genet 14: 146–151

Koehler CM, Lindberg GL, Brown DR, Beitz DC, Freeman AE, Mayfield JE, Myers AM (1991) Replacement of bovine mitochondrial DNA by a sequence variant within one generation. Genetics 129:247–255

Lockson D, Zweifel S, Freeman-Cook L, Lorimer H, Brewer B, Fangman W (1995) A role for recombination junctions in the segregation of mitochondrial DNA in yeast. Cell 81: 947–955

Marchington D, Poulton J, Seller A, Holt I (1996) Do sequence variants in the major non-coding region of the mitochondrial genome influence mitochondrial mutations associated with disease. Hum Mol Genet 5:473–479

Matthews PM, Hopkin J, Brown RM, Stephenson JB, Hilton-Jones D, Brown GK (1994) Comparison of the relative levels of the 3243 (A--G) mtDNA mutation in heteroplasmic adult and fetal tissues. J Med Genet 31:41–44

Monckton DG, Neumann R, Guram T, Fretwell N, Tamaki K, MacLeod A, Jeffreys AJ (1994) Minisatellite mutation rate variation associated with a flanking DNA sequence polymorphism. Nat Genet 8:162–170

Monnat R, Loeb L (1985) Nucleotide sequence preservation of human mitochondrial DNA. Proc Natl Acad Sci USA 82: 2895–2899

Poulton J, Morten K (1993) Noninvasive diagnosis of the MELAS syndrome from blood DNA. Ann Neurol 34:116

Poulton J, O'Rahilly S, Morten K, Clark A (1995) Mitochondrial DNA, diabetes, and pancreatic pathology in Kearns-Sayre syndrome. Diabetologia 38:868–871

Solignac M, Genermont J, Monnerot M, Mounolou J (1987) Drosophila mitochondrial genetics: evolution of heteroplasmy through germ line cell divisions. Genetics 117:687–696

Yoneda M, Chomyn A, Martinuzzi A, Hurko O, Attardi G (1992) Marked replicative advantage of human mtDNA carrying a point mutation that causes the MELAS encephalomyopathy. Proc Natl Acad Sci USA 89:11164–11168